The Human Papillomavirus E6 protein and its contribution to malignant progression

Fiamma Mantovani[1] and Lawrence Banks*,[1]

[1]*International Centre for Genetic Engineering and Biotechnology Padriciano 99, I-34012 Trieste, Italy*

The Human Papillomavirus (HPV) E6 protein is one of three oncoproteins encoded by the virus. It has long been recognized as a potent oncogene and is intimately associated with the events that result in the malignant conversion of virally infected cells. In order to understand the mechanisms by which E6 contributes to the development of human malignancy many laboratories have focused their attention on identifying the cellular proteins with which E6 interacts. In this review we discuss these interactions in the light of their respective contributions to the malignant progression of HPV transformed cells. *Oncogene* (2001) **20**, 7874–7887.

Keywords: HPV; E6; transformation; proteasome; PDZ domains

Introduction

Human Papillomaviruses (HPVs) infect keratinocytes in the basal layers of stratified epithelia at a variety of anatomical sites and their replicative cycle is intimately linked with the differentiation of the infected cell. Mucosal HPVs are the best characterized, and include the high-risk types, such as HPV-16 and HPV-18, which cause lesions that can progress to cervical carcinoma. In contrast, the low-risk types, such as HPV-6 and HPV-11, induce benign genital warts and are very rarely associated with malignancies (zur Hausen and Schneider, 1987). Less is known about the cutaneous HPV types but, once again, a subset of these types, including HPV-5 and HPV-8, is linked with the development of human cancers, particularly squamous cell carcinoma (SCC) at sun exposed sites in immunocompromised individuals (for review see Benton and Arends, 1996).

Due to their limited coding capacity, HPVs have to use the cellular DNA synthesis machinery in order to replicate their genomes. However, while low-risk HPVs begin replication in cells that are still proliferating, the replicative phase of high-risk HPV infection is confined to more differentiated cells that have already exited the cell cycle and are non-permissive for DNA synthesis (Doorbar *et al.*, 1997). In order to overcome this

problem, the high-risk HPV E7 protein targets a number of cell cycle regulatory proteins, including the 'pocket protein' family of pRb, p107 and p130, thereby upregulating genes required for G1/S transition and DNA synthesis (see Münger *et al.*, this issue). However, the host cell's normal response to this unscheduled induction of proliferation would be to trigger apoptosis and/or growth arrest. To overcome these obstacles, the high-risk E6 protein targets a variety of cellular proteins involved in regulating these defence mechanisms, as well as those involved in terminal differentiation and antiviral defence. Under normal circumstances, viral replication would then continue, resulting in production and release of infectious virions. On rare occasions, however, the viral life cycle is interrupted and processes are initiated that lead to immortalization and ultimately to full transformation of the cell.

In this review we shall address the functions of E6 which appear most relevant for cell transformation, highlighting those aspects which appear to be conserved across many different viral oncoproteins, as well as those reflecting unique aspects of E6 function. Prior to this we will review the studies which define the high-risk HPV E6 as a potent viral oncoprotein.

Transformation by HPV E6

The HPV E6 proteins are small polypeptides of approximately 150 amino acids and contain two zinc-finger motifs (Cole and Danos, 1987; Barbosa *et al.*, 1989), whose integrity is essential for E6 function (Kanda *et al.*, 1991; Sherman and Schlegel, 1996). The first indirect evidence that E6 was a viral oncoprotein came from studies on cervical tumours and derived cell lines, where E6 was found to be retained and expressed many years after the initial transforming events (Schwarz *et al.*, 1985; Androphy *et al.*, 1987; Banks *et al.*, 1987). Subsequently E6 was found to possess intrinsic transforming activity in a variety of different assay systems. Although E6 has weak transforming activity in established rodent cells (Sedman *et al.*, 1991), high-risk but not low-risk E6 proteins can efficiently cooperate with an activated ras oncogene in the transformation of primary rodent cells (Storey and Banks, 1993; Pim *et al.*, 1994; Liu *et al.*, 1994). In addition, E6 has also been found to immortalize

*Correspondence: L Banks; E-mail: Banks@icgeb.trieste.it

primary human mammary epithelial cells at late passage (Band *et al.*, 1991; Wazer *et al.*, 1995), although this activity is also exhibited by the low-risk HPV E6 proteins (Band *et al.*, 1993).

Perhaps the most relevant system for evaluating the transforming potential of the HPV oncoproteins is immortalization of primary human keratinocytes, which are the natural host cells of the virus *in vivo*. Numerous studies have shown that high-risk E6 and E7 are together sufficient to induce immortalization of primary human keratinocytes (Münger *et al.*, 1989; Hawley-Nelson *et al.*, 1989), while the low-risk HPV proteins are completely inactive in this assay (Wood-worth *et al.*, 1989; Pecoraro *et al.*, 1989). It is also interesting to note that these keratinocytes, although immortalized, will not form tumours in nude mice. Only following expression of activated oncogenes (DiPaolo *et al.*, 1989) or after extended passage in tissue culture (Dürst *et al.*, 1989; Hurlin *et al.*, 1991) do these cells become fully transformed. This nicely resembles the process of HPV induced tumorigenicity *in vivo*, where there are long periods between the initial immortalization events and the ultimate progression to cervical cancer, thereby highlighting the multistep nature of the disease progression.

More recently, efficient models of HPV-induced carcinogenesis have been obtained through the generation of transgenic mice. E6 and E7 together can induce various types of tumours, depending on the particular tissue in which they are expressed (Arbeit *et al.*, 1993; Griep *et al.*, 1993; Comerford *et al.*, 1995): when targeted to the basal cells of the squamous epithelium, under control of the human keratin 14 promoter (K14), the transgenic mice developed progressive squamous epithelial neoplasia (Arbeit *et al.*, 1994). Individual expression of each oncogene induced epithelial hyperplasia and skin tumours (Herber *et al.*, 1996; Song *et al.*, 1999), however E7 was found to primarily cause benign, highly differentiated tumours, whereas those promoted by E6 were mostly malignant. This suggests that the two oncoproteins play different roles in the process of carcinogenesis, and also supports the notion that they act cooperatively to induce transformation. Further investigation was performed on E6- and E7-transgenic mice, following treatment with specific carcinogens known to affect distinct stages of tumour formation. Interestingly, E7 was found to primarily cause tumour promotion, whereas E6 contributed weakly to the early stages, acting more strongly during tumour progression, accelerating the malignant conversion of benign tumours (Song *et al.*, 2000): this finding was particularly important, since it suggests that E6 may be responsible for the malignant progression of HPV induced tumours *in vivo*.

Interactions between HPV E6 and p53

Analysis of the cellular targets of the HPV E6 proteins has provided a wealth of information on how E6 contributes to malignant transformation. The first cellular target of E6 to be identified, and probably still the most important, is p53.

The p53 tumour suppressor represents a major constraint to viral replication, since, once activated by the unscheduled induction of DNA replication, it can promote cell cycle arrest or apoptosis of the infected cell (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Lowe *et al.*, 1994; Wu and Levine, 1994). To overcome this obstacle, several viruses encode proteins that functionally inactivate p53. SV40 LT prevents transactivation of p53 target genes through association with its DNA binding domain (Ruppert and Stillmann, 1993), Ad E1B-55K abolishes the same function by binding to the transactivating domain of p53 (Lin *et al.*, 1994), yet in association with E4orf6 it can also lead to p53 degradation (Steegenga *et al.*, 1998, Querido *et al.*, 2001; Täuber and Dobner, this issue), while the HBV X protein sequesters p53 in the cytoplasm (Elmore *et al.*, 1997). A major strategy employed by the high-risk HPV E6 proteins to abrogate p53's oncosuppressive functions is to induce its degradation through the ubiquitin-proteasome pathway (Scheffner *et al.*, 1990). As a consequence, p53 levels are extremely low in cervical tumour cells (Matlashewski *et al.*, 1986), and p53-induced growth arrest and apoptosis in response to DNA damage are abolished (Kessis *et al.*, 1993; Foster *et al.*, 1994). Under normal growth conditions in the absence of HPV, p53 is also turned over by the ubiquitin proteasome pathway, and this is mediated by the ubiquitin ligase Mdm2 (Honda *et al.*, 1997). However under stress conditions, e.g. the expression of viral oncogenes, this degradative pathway is inhibited and p53 is both stabilized and activated (Ashcroft and Vousden, 1999, for review). Recent experiments have indeed shown that in HPV-positive cancer cells the Mdm2 pathway is completely inactive, while p53 degradation depends entirely on E6 (Hengstermann *et al.*, 2001). This indicates that E6 can target p53 for degradation under conditions when this would be normally inhibited, e.g. after DNA damage, thereby allowing the accumulation of genomic mutations in the infected keratinocytes, and thus contributing towards malignant progression. E6 reactivates degradation of p53 by recruiting E6AP, a ubiquitin ligase. E6AP belongs to the HECT-domain family of ubiquitin ligases, whose large and divergent N-terminal domains mediate substrate recognition, while ubiquitination of bound substrates is catalysed by the conserved C-terminal HECT domain (Schwarz *et al.*, 1998). High-risk HPV E6 binds to E6AP within its N-terminal substrate recognition domain (Huibregtse *et al.*, 1993a), and formation of a stable E6–E6AP complex precedes association with p53, thereby redirecting the substrate specificity of E6AP towards p53 (Huibregtse *et al.*, 1993b). Indeed, approaches aimed at blocking E6AP activity, either by the use of antisense oligonucleotides (Beer-Romero *et al.*, 1997) or dominant negative mutants (Talis *et al.*, 1998), increased the levels of p53 in HPV-positive, but not in HPV-negative cells,

confirming that E6AP plays an essential role in E6 directed degradation of p53 *in vivo*, but has no effect on p53 levels in cells lacking E6.

The efficiency in mediating degradation of p53 varies among different E6 proteins, depending on their ability to interact with both p53 and E6AP. Both high- and low-risk mucosal HPV E6 proteins are able to bind the p53 C-terminus, however such interactions do not induce degradation. Binding to the core region of p53 is much greater for high-risk E6 proteins and is enhanced by the presence of E6AP, and it is this interaction that allows efficient degradation of p53 (Li and Coffino, 1996). In addition, HPV-16 E6 binds E6AP more strongly, and concomitantly degrades p53 more effectively, than HPV-18 E6. HPV-11 E6 has minimal levels of binding to E6AP (Huibregtse *et al.*, 1993b), and degrades p53 *in vivo* only weakly (Storey *et al.*, 1998). Interestingly, the E6 proteins of both high- and low-risk cutaneous HPV types do not associate with either E6AP or p53 and are incapable of affecting p53 stability (Elbel *et al.*, 1997), therefore the mechanism by which these viruses evade the constraints that p53 places on viral replication remain to be determined.

Although targeting p53 for degradation is the major route by which E6 overcomes its effects, several reports indicate that E6 makes use of additional pathways to abrogate p53's growth suppressive activities. Both low- and high-risk HPV E6 proteins are capable of abolishing p53-mediated transcriptional repression *in vivo* (Lechner *et al.*, 1992) and this is likely to occur through binding to the p53 C-terminus (Li and Coffino, 1996). Moreover, the capacity of the high-risk E6 proteins to abrogate transactivation of p53 target genes does not only depend on p53 destabilization, since E6 mutants defective for degradation retain the ability to abrogate transcriptional activation by p53 *in vivo* (Pim *et al.*, 1994). Several mechanisms can be invoked to explain this property of E6. First, E6 can interfere with binding of p53 to its DNA recognition site (Lechner and Laimins, 1994; Thomas *et al.*, 1995). In addition, repression of p53-responsive promoters could be mediated through the interaction of high-risk HPV-16 E6 with the transcriptional coactivators p300/CBP (Patel *et al.*, 1999; Zimmermann *et al.*, 1999), similarly to what has been reported for Ad E1A (Somasundaram and El-Deiry, 1997). Finally, cytoplasmic sequestration of p53 is another common strategy adopted by different viral proteins, such as Ad E1B 55K (König *et al.*, 1999), HBV protein X (Elmore *et al.*, 1997) and HPV E6. It has been reported that in HPV-positive cancer cells, the nuclear localization of p53 in response to DNA damage is blocked even if proteasome degradation is inhibited (Mantovani and Banks, 1999). Cytoplasmic retention may be due to masking p53's nuclear localization signal by E6 binding to the p53 C-terminus, or to enhanced nuclear export of p53. The latter mechanism is supported by the observation that inhibition of nuclear export in HPV-positive tumour cells by the drug leptomycin B results in accumulation of p53, indicating that E6-mediated degradation of p53 is, at least in part,

dependent on nuclear export (Freedman and Levine, 1998). Although these data strongly suggest that it occurs in cytoplasmic proteasomes, colocalization of the two proteins in the cytoplasm is not alone sufficient to trigger degradation. In neuroblastoma cells p53 is constitutively cytoplasmic and appears to be resistant to proteolysis induced by either Mdm2 or E6 (Isaacs *et al.*, 1999), implying the requirement for nucleocytoplasmic shuttling to allow degradation. Whether this process is mediated directly by E6, its cellular partner E6AP, or another protein, however remains to be determined, since E6, E6AP and p53 all contain putative nuclear export signals.

It is quite clear, however, that during viral infection and in HPV-induced cervical lesions not all p53 is degraded, as several studies have reported detectable levels of p53 in HPV-infected cells (Mantovani and Banks, 1999; Cooper *et al.*, 1993; Lie *et al.*, 1999). A possible viral pathway for regulating E6 activity with respect to p53 relies on a series of polypeptides termed E6*, which are expressed by the high-risk HPV types through alternative splicing of E6 mRNA (Schneider-Gädicke *et al.*, 1988). Interestingly, HPV-18 E6*I was found to interact with both the full-length E6 and E6AP, thereby blocking degradation of p53 (Pim *et al.*, 1997): this might allow a fine-tuning of the activity of E6 with respect to p53 during viral infection. Interestingly, while p53 has been shown to specifically inhibit HPV amplificational DNA replication *in vivo*, it did not affect episomal maintenance, which occurs in synchrony with the cell cycle (Lepik *et al.*, 1998). In order to elicit a productive infection, viral DNA amplification needs to be controlled and it is plausible that the activity of E6* could ensure the presence of a limited amount of p53 at the replication sites, where it could both prevent overreplication of the viral genome and, possibly, assist DNA synthesis by means of its proofreading capacity. Indeed, HPV recruits DNA polymerase α for DNA replication, and the $3' - 5'$ exonuclease activity of p53 could enhance the replicative fidelity of this enzyme (for review, Albrechtsen *et al.*, 1999). It is interesting to note that p53 has been found in the replication centres of Herpes Simplex Virus (Wilcock and Lane, 1991), Cytomegalovirus (Fortunato and Spector, 1998) and Adenovirus (König *et al.*, 1999), and recent studies reported an interaction between the HPV ori-complex binding protein E2 and p53, further suggesting a potential positive role for p53 in viral replication (Massimi *et al.*, 1999).

It is quite clear that the E6 – p53 interaction represents one of the key events in E6 induced malignancy: continuous degradation of p53 can lead to the accumulation of genetic mutations in the infected cell. Indeed, loss of p53 leads to early tumour development (Donehower *et al.*, 1992), and enhances the malignant progression of chemically induced skin cancers in mice (Kemp *et al.*, 1993), which is consistent with the observation that E6 accelerates the malignant conversion of tumours promoted by E7 (Song *et al.*, 2000). In addition, E6 has been shown to induce gross chromosomal alterations in cell culture, including

translocations and aneuploidy (Reznikoff *et al.*, 1994; White *et al.*, 1994). These chromosomal changes are also common in malignant cells that have lost p53 function, indicating that E6 can induce genomic instability through inactivation of p53.

p53-independent activities of E6

Although p53 is a vital aspect of E6 function, analysis of E6 mutants has shown that activities other than targeting p53 are required for its full transforming potential (Pim *et al.*, 1994; Nakagawa *et al.*, 1995; Liu *et al.*, 1999). Moreover, the oncogenic potential of cutaneous HPVs mainly relies on their E6 proteins which, however, do not interact with p53 (Elbel *et al.*, 1997). It is now clear that, in common with many viral oncoproteins, E6 is multifunctional and, in line with the above observations, numerous cellular targets have been identified, some of which are listed in Table 1 and Figure 1. There is now active debate as to which of these other activities of E6 are relevant for the development of malignancy.

De-regulation of transcription and DNA replication by HPV E6

The E6 proteins of both high- and low-risk HPV types have long been known to modulate transcription from many cellular and viral promoters (Sedman *et al.*, 1991; Desaintes *et al.*, 1992; Etscheid *et al.*, 1994; Veldman *et al.*, 2001). It is only recently, however, that clues to the mechanisms by which this may occur have come, with the demonstration that E6 interacts with p300/CBP (Patel *et al.*, 1999; Zimmermann *et al.*, 1999). The p300/CBP transcriptional co-activators play important roles in activating a great number of genes involved in the regulation of cell cyle, differentiation and immune response. Indeed, many viral oncoproteins including SV40 LT, Py LT, EBNA2, Ad E1A, and HTLV-1 Tax have been shown to require the interaction with p300 for optimal transforming activity (Goodman and Smolik, 2000,

for review), highlighting its central importance in regulating cellular homeostasis. HPV-16 E6 was shown to directly bind three regions of p300/CBP, namely C/H1, C/H3 and the carboxy terminus, while low-risk HPV-6 E6 was found to only weakly bind to the C/H1 region (Patel *et al.*, 1999). Moreover, HPV-16 E6 was also shown to inhibit the intrinsic transcriptional activity of p300/CBP on both p53- and NF-κB-responsive promoter elements. Obviously, p300/CBP affects expression of many different cellular promoters, including those regulating differentiation (Bannister and Kouzarides, 1995; Goodman and Smolik, 2000), and at present it is not clear which are the true targets for E6 mediated inhibition via the p300/CBP interaction. However, possible E6 inhibition of NF-κB-responsive promoters is particularly intriguing, since dysregulation of the NF-κB pathway can result in hyperproliferation of the stratum spinosum, the epithelial layer in which HPV DNA amplification occurs (Hu *et al.*, 1999). NF-κB is activated upon viral infection and promotes transcription of a number of genes involved in the local immune response such as class I MHC, interleukins and GM$-$CSF, some of which are synthesized directly by the keratinocytes (for review see Baldwin, 1996), therefore inhibition of NF-κB target genes may help the virus to escape immune recognition. Moreover, NF-κB activation by viral infection stimulates the IFN-β promoter (Thanos and Maniatis, 1995), and thus E6 inhibition of NF-κB could help overcome the interferon-mediated antiviral response. Interestingly, HPV-16 E6 has also been found to bind the interferon regulatory factor 3 (IRF-3) (Ronco *et al.*, 1998), which is an important transactivator of interferons and binds to the regulatory elements of the IFN-β promoter (Wathelet *et al.*, 1998). As a consequence of this interaction, E6 was also found to inhibit IFN-β induction (Ronco *et al.*, 1998). Interestingly, a recent screen of cDNA microarrays demonstrated that transfection of HPV-16 E6 in differentiating cervical keratinocytes also downregulates the expression of interferon-responsive genes (Nees *et al.*, 2001). Since viral infection stimulates the assembly and activation

Table 1 Cellular targets of HPV E6

| Target protein	High-risk E6	Low-risk E6	Degradation	Cellular function	E6AP involved
p53	yes	yes	yes	Transcription factor, apoptosis inducer, tumour suppressor	yes
E6AP	yes	no	yes	Ubiquitin ligase	yes
hMcm7	yes	yes	yes	DNA replication initiation	yes
E6TP1	yes	yes	yes	Putative GAP protein	yes
Bak	yes	yes	yes	Apoptosis inducer	yes
c-Myc	yes	no	yes	Transcription factor	yes
p300/CBP	yes	yes	no	Transcriptional coactivators	no
AMF-1/Gps2	yes	yes	yes	Transcriptional coactivator	?
IRF-3	yes (HPV-16)	no	no	Transcription factor	no
E6BP/ERC-55	yes	no	no	Ca^{2+} binding protein	yes
paxillin	yes	no	no	Signal transduction	no
hDlg	yes	no	yes	Control of cell polarity and growth	no
MAGI-1	yes	no	yes	Putative signal transduction	no
MUPP-1	yes	no	yes	Scaffolding protein/putative signal transduction	no
hScrib	yes	no	yes	Control of cell polarity and growth	yes
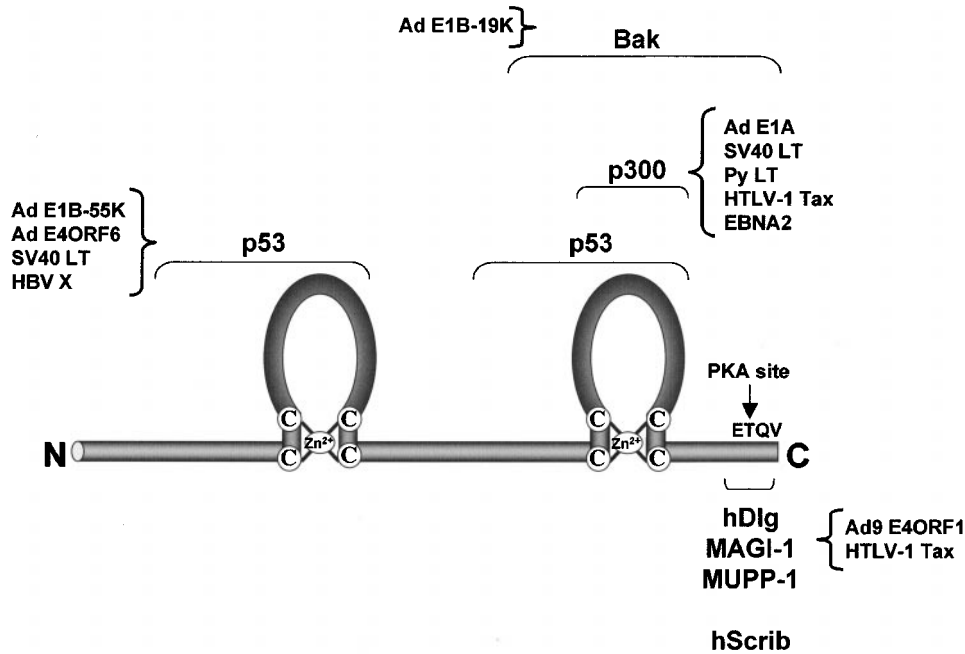
Figure 1 The high-risk HPV E6 protein. Schematic diagram of E6 showing the two zinc fingers, together with the regions involved in interactions with cellular proteins that are targeted by the oncoproteins of other viruses. Also shown is the C-terminal PDZ binding motif, ETQV, and the overlapping site of PKA phosphorylation is arrowed

of a large transcription factor complex that includes IRF-3 and CBP/p300, which in turn activates α/β interferon-responsive genes (Weaver *et al.*, 1998), it is likely that the combined targeting of all these components by E6 will contribute to the disruption of the cellular antiviral response.

Finally, it should also be borne in mind that the E6/p300 interaction, and its possible contribution towards survival of the transformed cell, could be a by-product of the regulation of viral gene expression. Thus, the HPV major transcriptional activator, E2, also interacts with p300/CBP (Lee *et al.*, 2000a; Marcello *et al.*, 2000). This appears to involve a cellular protein, AMF-1/Gps2, which in turn enhances p300 activity but which, intriguingly, is also a target for E6 mediated degradation (Peng *et al.*, 2000; Degenhardt and Silverstein, 2001). Therefore the interaction between E6 and p300 may represent a means of downregulating E2 transcriptional activity, and thereby controlling the levels of E6 expression by a feed-back mechanism.

An additional link with tumour progression and HPV E6 activity has come from studies showing deregulation of the cellular DNA replication machinery. Normal somatic cells terminate their replicative life span through a pathway leading to cellular senescence, which is triggered in response to critically shortened telomere DNA. Telomerase activity is absent from most normal somatic cells and neoplastic cells must first overcome the senescence checkpoint mechanisms, and subsequently activate telomerase to propagate indefinitely. In senescent cells the levels of p16^{ink} are elevated, and

its inactivation has been shown to abrogate a late step during senescence of human epithelial cells (Kiyono *et al.*, 1998). Interestingly, the immortalization of human uroepithelial cells by HPV-16 E6 correlated with undetectable levels of p16^{ink} due to gene inactivation. In addition, activation of telomerase is detected in cervical carcinomas and in a subset of high grade cervical lesions associated with high-risk HPV (Snijders *et al.*, 1998). Telomerase can be induced by HPV-16 E6 in primary epithelial cells through a p53-independent mechanism (Klingelhutz *et al.*, 1996; Veldman *et al.*, 2001) via E6-mediated transcriptional activation of the gene encoding the telomerase catalytic subunit, hTERT, and the minimal promoter region involved in induction by E6 was found to require an intact E box (Veldman *et al.*, 2001; Gewin and Galloway, 2001). Interestingly, this activation of the hTERT promoter was found to be dependent on the ability of E6 to interact with E6AP, suggesting that the activity of the E6-E6AP complex may target a regulator of the hTERT promoter (Gewin and Galloway, 2001).

E6 proteins of both benign and oncogenic HPV types have also been found to interact with hMcm7, a component of the DNA replication licensing complex, suggesting that this interaction might be required for viral genome replication (Kukimoto *et al.*, 1998; Kühne and Banks, 1998). However, hMcm7 is a substrate for E6AP-dependent ubiquitination, and HPV-18 E6 was able to enhance its proteasome degradation *in vivo* (Kühne and Banks, 1998): this might also be expected to cause p53-independent chromosomal abnormalities in HPV-positive cells.

Mitogenic activities of E6

It has been shown that expression of oncogenic E6 can affect the early stages of carcinogenesis in transgenic mice, although to a much lesser extent than E7 (Song *et al.*, 2000), and this probably relies on the ability of E6 to induce cellular hyperproliferation and epidermal hyperplasia (Song *et al.*, 1999). Interestingly, this appears to be largely independent of p53, since similar levels of proliferation are induced by E6 in p53-null mice (Song *et al.*, 1999). At present, little is known about the pathways by which E6 could stimulate proliferation but a recent study identified E6TP1 in a two-hybrid screen. This protein shows high homology with a family of GTPase activating proteins (GAPs) that are negative regulators of Rap (Gao *et al.*, 1999). Therefore E6TP1 might be involved in the inhibition of Rap-mediated mitogenic signalling. Moreover, its gene has been mapped to a putative tumour suppressor locus on chromosome 14 (Menon *et al.*, 1997). High-risk and, to a lesser extent, low-risk HPV E6 proteins can bind E6TP1, while only the oncogenic E6s can promote its degradation. Interestingly, E6AP was reported to participate in the interaction, and it might therefore be responsible for ubiquitination of E6TP1 (Gao *et al.*, 1999). There is a strong correlation between the ability of HPV-16 E6 mutants to degrade E6TP1 and to immortalize human mammary epithelial cells (Gao *et al.*, 2001), however more data concerning the GAP function of E6TP1 are necessary to understand its role in E6-induced malignancy.

Inhibition of apoptosis

Oncogenic HPV E6 proteins counteract the induction of apoptosis by activated p53 by inducing its degradation. However, there is increasing evidence that E6 can also inhibit p53-independent apoptotic pathways, promoted by different stimuli. Indeed, in transgenic mice expressing HPV-16 E6 in the ocular lens, E6 was found to prevent apoptosis both in wt and in p53-null animals (Pan and Griep, 1995), and it has also been reported to inhibit drug-induced apoptosis in cells lacking p53 (Steller *et al.*, 1996). Among the more recently discovered cellular targets of E6, there are several proapoptotic factors. One of them is Bak, whose high levels of expression in the upper epithelial layers (Krajewski *et al.*, 1996) appear to represent a common obstacle for a broad range of HPV types that replicate in differentiating keratinocytes, since the E6 proteins of both high and low-risk mucosal HPVs, plus those of high-risk cutaneous types, have been shown to inhibit Bak-induced apoptosis (Thomas and Banks, 1998, 1999; Jackson and Storey, 2000). HPV-18 E6 stimulates the ubiquitin-dependent degradation of Bak catalysed by E6AP, probably by accelerating a normal cellular process, since Bak appears to be a substrate of E6AP in the absence of E6 (Thomas and Banks, 1998). Degradation of Bak by HPV-11 E6 is less effective, and this correlates with a weaker anti-apoptotic activity of the low-risk mucosal HPV types (Thomas and Banks,

1999). Recently, a link was demonstrated between cutaneous HPV infection and the induction of HPV-associated skin cancer by UV radiation. The cutaneous E6 proteins were shown to abrogate both p53-dependent and independent apoptosis in response to UV-induced DNA damage. p53 protein levels were unaffected in cells expressing cutaneous E6s, and its transcriptional activity with respect to p21 also did not change (Jackson and Storey, 2000). The mechanism used by these E6 types to inhibit p53-dependent apoptosis is not known, although one possibility is that they mediate the specific inhibition of p53 target genes directly involved in apoptosis, such as the PIG genes induced by DNA damage (Polyak *et al.*, 1997). The cutaneous HPV E6 proteins also abrogate Bak function by promoting its ubiquitin-mediated degradation and Bak protein is undetectable in HPV-positive skin cancers, in contrast to HPV negative cancers which express it (Jackson *et al.*, 2000). Interestingly, Ad E1B 19K also inhibits Bak induced apoptosis (Farrow *et al.*, 1995; White, this issue), demonstrating a high degree of conservation of function among these viruses.

The c-Myc transcription factor is normally degraded through the proteasome pathway, and it was shown that high-risk, but not low-risk HPV E6, accelerates degradation of c-Myc by recruiting E6AP (Gross-Mesilaty *et al.*, 1998). It may seem unusual that E6 targets a protein which would normally stimulate cell proliferation, but this finding can be also viewed in the light of c-Myc proapoptotic activity. TGFβ is a potent growth inhibitor for many cell types, including most epithelial cells (Lyons and Moses, 1990), and its effects are mediated at least in part by suppression of c-*myc* transcription (Pietenpol *et al.*, 1990a). It has been demonstrated that HPV-16 E7 and other viral proteins binding pRb, such as SV40 LT and Adenovirus E1A, are able to block TGFβ-mediated downregulation of c-Myc, in order to sustain proliferation of the infected keratinocytes (Pietenpol *et al.*, 1990b; Münger *et al.*, this issue). However, deregulated expression of c-Myc in differentiating cells induces apoptotic cell death (Askew *et al.*, 1991). It is therefore possible that degradation of c-Myc by E6 contributes towards maintaining an equilibrium between E7-promoted proliferation and cell survival.

Interference with epithelial organization and differentiation

High-risk HPV types have evolved to replicate in the differentiated layers of the squamous epithelium, in an environment that is non-permissive for DNA replication. A characteristic of the high-risk E6 proteins is their ability to inhibit terminal differentiation of epithelial cells, which normally leads to keratinization and cell death. HPV-16 E6 impairs cell differentiation in the ocular lens of transgenic mice (Pan and Griep, 1994) and causes expansion of the undifferentiated compartment of the epithelia in K14-E6 transgenic mice and, interestingly, this activity of E6 appears to

be p53-independent (Song *et al*., 1999). Consistent with this, HPV-16 E6 was also found to increase the resistance of human keratinocytes to serum and calcium induced differentiation through p53-independent pathways (Sherman *et al*., 1997; Sherman and Schlegel, 1996), although little is known about the molecular mechanisms by which E6 does this. However, HPV-16 E6 was reported to interact with E6BP/ ERC-55 (Chen *et al*., 1995) which is a putative calcium binding protein localized in the endoplasmic reticulum (Weis *et al*., 1994). E6BP was found to form a complex with both E6 and E6AP *in vivo*, however direct binding to E6AP and E6 targeted degradation were not shown (Chen *et al*., 1995). Epithelial differentiation is responsive to Ca^{2+} mediated signalling, and it might be speculated that targeting E6BP contributes to E6's impairment of terminal differentiation. Ca^{2+} signalling also has a role in blocking apoptosis, and it is interesting that the antiapoptotic Ad E1B 19K protein also interacts with a putative calcium binding protein localized in the nuclear envelope and ER (Boyd *et al*., 1994). Hence, the E6–E6BP interaction might similarly be involved in the p53-independent inhibition of apoptosis in HPV infected cells.

Cellular adhesion to the extracellular matrix affects many different cellular processes including cell morphology, proliferation and migration. The restriction of cell proliferation to matrix–interacting cells serves to prevent dysplasia, and the circumvention of anchorage dependence plays an important role in tumorigenesis (Sastry and Horwitz, 1996). Cell–matrix adhesion is mediated by specialized structures called focal adhesions, which contain integrins, vinculin, focal adhesion kinase and paxillin (Sastry and Burridge, 2000). Paxillin is involved in mediating signalling from the plasma membrane to focal adhesions and to the actin cytoskeleton, and its activity is regulated by tyrosine phosphorylation in response to various stimuli, including integrin crosslinking and treatment with growth factors (Turner, 2000). HPV-16 E6 has also been shown to bind paxillin and this interaction correlates with E6 transforming activity, although it does not lead to paxillin degradation (Tong and Howley, 1997). However, since most of the biological effects of this interaction have been determined for the more highly abundant BPV-1 E6 protein, its relevance for high-risk HPV E6 proteins remains to be determined.

Interactions with PDZ proteins: interference with cell–cell adhesion, polarity and proliferation control

A striking feature of all E6 proteins derived from the high-risk HPV types is the presence of a highly conserved C-terminal domain (see Figure 1), which is not involved in p53 binding and degradation (Crook *et al*., 1991; Pim *et al*., 1994), but which nonetheless contributes to E6 transforming activity, since its deletion impairs E6's ability to transform rodent cells (Kiyono *et al*., 1997) and immortalize keratinocytes (C Meyers, personal communication). This region contains a PDZ-binding motif (XT/SXV), a short stretch

of amino acids which mediates the specific interaction with proteins containing PDZ domains (Doyle *et al*., 1996; Songyang *et al*., 1997). These are 80–90 amino-acid long motifs, present on a variety of proteins involved in clustering ion channels, signalling enzymes, and adhesion molecules to specific structures at the membrane-cytoskeleton interface of polarized cells (reviewed in Kim, 1997). The first PDZ-protein shown to be a target for high-risk E6 was hDlg (Kiyono *et al*., 1997; Lee *et al*., 1997). This is the human homologue of the *Drosophila* tumour suppressor Dlg, required for formation of adherens junctions and for regulation of cell adhesion, apicobasal polarity and proliferation in epithelial tissues. Indeed, loss of Dlg function causes aberrant morphology and invasive growth of epithelial cells, resulting in embryonic lethality (Woods *et al*., 1996; Bilder *et al*., 2000). A recent study also described a *Dlg* truncation mutant that resulted in impaired morphogenesis and perinatal death during murine development (Caruana and Bernstein, 2001). Human hDlg colocalizes with E-cadherin at adherens junctions of epithelial cells, (Reuver and Garner, 1998; Ide *et al*., 1999) and interacts through different domains (see Figure 2) with several proteins, including Shaker-type K^+ channels (Kim *et al*., 1995), cytoskeletal protein 4.1 (Lue *et al*., 1994; Marfatia *et al*., 1996), and the APC tumour suppressor protein (Matsumine *et al*., 1996), which is mutated in the majority of colon cancers (Kinzler and Vogelstein, 1996). Indeed, complex formation between hDlg and APC was reported to block cell cycle progression (Ishidate *et al*., 2000). HPV E6 can target hDlg for ubiquitin mediated degradation (Gardiol *et al*., 1999), probably by enhancing a physiological process, since hDlg appears to be ubiquitinated and degraded by the proteasome in cells even in the absence of E6 (Gardiol *et al*., 1999; Mantovani *et al*., 2001, in press). This interaction between E6 and hDlg might be necessary at a defined point during the viral life cycle, in order to disrupt cell junctions and to abolish cell polarity, thereby altering the normal maturation and inducing proliferation of the infected keratinocytes. However, the consequences for the metastatic potential of tumours harbouring high-risk HPVs are clear. While HPV-18 E6 has a canonical PDZ-binding motif (ETQV), HPV-16 E6 has only a sub-optimal consensus site (ETQL). Consequently, HPV-18 E6 binds hDlg with higher affinity than HPV-16 E6 (Pim *et al*., 2000) and can degrade it more efficiently (Gardiol *et al*., 1999; Pim *et al*., 2000). Interestingly, this also correlates with the different malignant potential of the viruses, since it has been reported that cervical tumours associated with HPV-18 are more invasive and recurrent than those caused by HPV-16 (Burnett *et al*., 1992; Kurman *et al*., 1988; Zhang *et al*., 1995); this is in marked contrast with the reported lower efficiency with which HPV-18 E6 degrades p53 (Scheffner *et al*., 1990). Interestingly, low-risk E6 proteins lack PDZ-binding motifs, and indeed they can neither bind hDlg (Kiyono *et al*., 1997; Lee *et al*., 1997) nor induce its degradation (Gardiol *et al*., 1999; Pim *et al*., 2000), however they acquire this
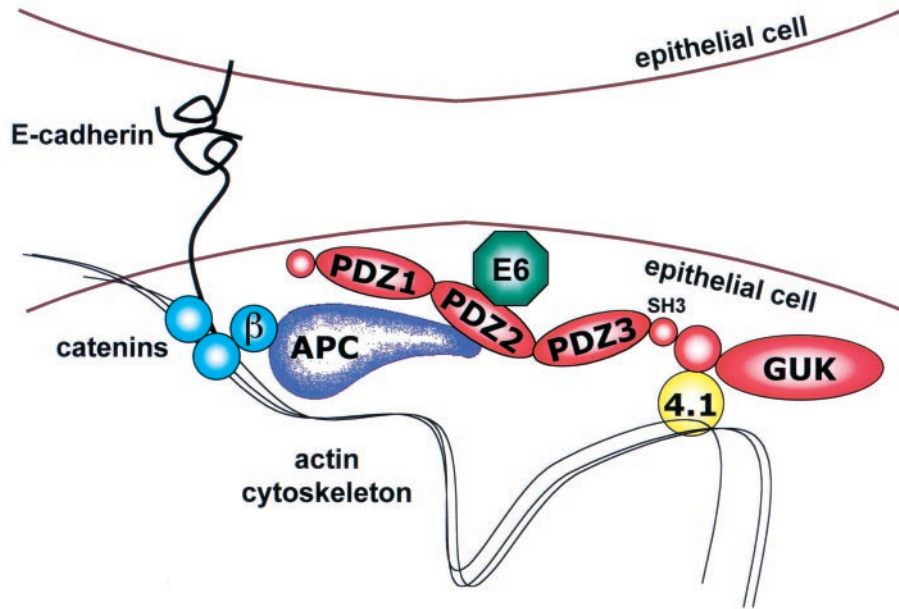
Figure 2 The hDlg protein. Schematic diagram showing the location of hDlg at the membrane-cytoskeleton interface of epithelial cells at regions of cell–cell contact. hDlg contains 3 PDZ domains, an SH3 signalling domain and a Guanylate Kinase (GUK) homology domain. Here it co-localizes with E-cadherin and binds the APC tumour suppressor, which in turn regulates β-catenin. The interaction between hDlg and APC is via PDZ domain 2 and this is the same domain targeted by the high risk HPV E6 proteins. Also shown is the interaction with protein 4.1, which connects hDlg to the actin cytoskeleton

ability if provided with a C-terminal PDZ binding domain derived from a high-risk E6 protein (Pim *et al.*, 2000). In contrast, cutaneous HPV E6 proteins are unable to degrade hDlg even when the interaction is allowed by adding a PDZ-binding motif to their C-termini (Pim *et al.*, submitted). This suggests that the mucosal HPV E6 proteins, whether derived from high or low-risk HPV types, can interact similarly with the cellular proteolytic machinery, whereas the high-risk cutaneous HPV E6 proteins appear to significantly differ in this aspect.

An increasing number of PDZ domain-containing proteins involved in the organization of epithelial architecture have now been reported to be targeted by E6 through their PDZ domains. Among them is hScrib, a protein expressed at epithelial tight junctions, which has recently been shown to be a substrate for ubiquitination by the E6-E6AP complex *in vitro*, and for proteasome degradation mediated by high-risk E6 *in vivo* (Nakagawa and Huibregtse, 2000). hScrib is the human homologue of *Drosophila* tumour suppressor Scrib, which cooperates with Dlg and Lgl to control both formation of cell junctions and inhibition of epithelial cell growth, possibly through controlling the localization of growth factor receptors and signalling molecules (Bilder and Perrimon, 2000; Bilder *et al.*, 2000). Interestingly, expression of oncogenic HPV E6 in mammalian cells was reported to disrupt the integrity of epithelial tight junctions, while E6 mutated in the PDZ-binding motif did not (Nakagawa and Huibregtse, 2000). This further suggests that combined degradation of different PDZ-proteins by E6 might be

responsible for the loss of epithelial cell adhesion and polarity of HPV-positive cancer cells. E6 proteins from high-risk, but not low-risk HPV types were also shown to bind another tight junction protein, MAGI-1 (Dobrosotskaya *et al.*, 1997; Ide *et al.*, 1999), through the first of its five PDZ domains (Thomas *et al.*, 2001), and to reduce its steady-state levels and half-life (Glaunsinger *et al.*, 2000). MAGI-1 forms a complex with β-catenin (Dobrosotskaya and James, 2000), the expression of which is deregulated in many human cancers (reviewed in Polakis, 1999). Interestingly, the closely related proteins MAGI-2 and MAGI-3 are involved in the regulation of the PTEN tumour suppressor, a component of the Akt kinase signalling pathway that promotes cell survival and proliferation (Marte and Downward, 1997): MAGI proteins are required to enhance PTEN's ability to suppress Akt activation, probably through assembling a multiprotein complex at the cell membrane (Wu *et al.*, 2000a,b). Therefore it is plausible that MAGI-2/3 degradation, promoted by high-risk HPV E6 (M Thomas, personal communication), might also affect Akt signalling and thereby inhibit apoptosis independently of targeting p53, as well as representing an alternative mitogenic activity of E6 (see above).

HPV-18 E6 has also been shown to bind MUPP1, a large multi-PDZ scaffold protein with a putative role in signal transduction (Ullmer *et al.*, 1998). Indeed, MUPP1 interacts selectively through its 10th PDZ domain with the cytoplasmic portion of the 5-HT$_{2c}$ serotonin receptor (Ullmer *et al.*, 1998; Becamel *et al.*, 2001), and with the unphosphorylated c-Kit tyrosine

kinase receptor (Mancini *et al.*, 2000). HPV-18 E6 was shown to reduce MUPP1 half-life *in vitro* and *in vivo* (Lee *et al.*, 2000b), and there is evidence that this also occurs through proteasome mediated degradation (F Mantovani, personal observations). Destruction of MUPP1 by E6 could thereby interfere with the assembly of signalling complexes at the epithelial cell membranes.

It has been frequently reported that different oncogenic viruses inactivate the same cellular targets to overcome common obstacles to their replication: thus, there are viral oncoproteins other than HPV E6 which bind PDZ-proteins, and this contributes to their transforming potential. hDlg has been shown to interact, through its PDZ domains, with both Ad 9 E4-ORF1 (Lee *et al.*, 1997; Täuber and Dobner, this issue) and HTLV-1 Tax, and these interactions interfere with the binding of APC, thereby perturbing cell growth control (Lee *et al.*, 1997; Suzuki *et al.*, 1999). MUPP1 and MAGI-1 are also bound by the Ad 9 E4-ORF1, which abolishes their activities by sequestering them into cytoplasmic bodies (Lee *et al.*, 2000b; Glaunsinger *et al.*, 2000). However, ubiquitin mediated degradation of PDZ-proteins is, to date, an exclusive property of the high-risk HPV E6 proteins. It has recently been shown that E6 can bridge the interaction between hScrib and the E6AP ubiquitin ligase, which normally would not recognize hScrib (Nakagawa and Huibregtse, 2000). This is reminiscent of p53 degradation, yet a clear role for E6AP in hScrib degradation *in vivo* has not been confirmed. In contrast, proteasome degradation of hDlg appears to be independent of E6AP (Pim *et al.*, 2000), as deduced from the basis of E6's ability to degrade hDlg in extracts lacking E6AP, and from the fact that E6 mutants defective for E6AP binding and p53 degradation can still degrade hDlg. Similarly, low-risk E6 proteins can also target hDlg for degradation, with an efficiency similar to that of the high-risk E6 proteins, when they are provided with a PDZ-binding motif (Pim *et al.*, 2000). However, these low risk E6 proteins show only minimal levels of interaction with E6AP (Huibregtse *et al.*, 1991, 1993b).

E6 binding motifs: a matter of specificity

It is clear from the above discussion that E6 is a multifunctional protein which efficiently interferes with diverse cellular pathways. However, it is logical to ask how such a small and low abundance viral protein could evolve to interact with so many different cellular proteins, both with respect to the specificity of the interactions and to the relatively high abundance of the partners. The analysis of E6 targets has identified conserved E6 binding motifs, thereby characterizing clusters of proteins which are bound by E6 through similar domains. The PDZ domain, for instance, defines a family of proteins with common functions, which are targeted by high-risk E6 proteins through

their C-terminal PDZ-binding motifs. E6 interaction with proteins containing multiple PDZ domains is, however, highly specific: thus, despite their high overall homology, only single PDZ domains on hDlg and MAGI-1 (Kiyono *et al.*, 1997; Thomas *et al.*, 2001) are recognized by the E6 protein. Another conserved binding site, the L2G box which comprises a α-helical motif, has been found on a number of cellular targets of E6, including E6AP, hMcm7, E6BP and paxillin. The binding specificity of E6AP is mediated by this motif, present on both enzyme and substrate, and this in turn is used by E6 to interact with both (Chen *et al.*, 1998; Elston *et al.*, 1998; Kühne and Banks, 1998). Not surprisingly, E6 has also been reported to induce self-ubiquitination of E6AP (Kao *et al.*, 2000), nonetheless, there are proteins which interact with E6 through an L2G box without being targeted for degradation, such as E6BP (Chen *et al.*, 1995) and paxillin (Tong and Howley, 1997).

The amount of the E6 protein produced during a viral infection is very low, and the problem of targeting cellular proteins which are much more abundant within the cell is efficiently solved by inducing their ubiquitin-mediated degradation. Moreover, it seems plausible that the virus does not require the complete destruction of its targets, rather, even a transient decrease of their local concentration within the cell is likely to perturb the physiological conditions in favour of viral replication. This also implies that E6 does not need to interact with all of its putative targets at the same time, since probably only a limited subset will be available at defined stages during cell differentiation and in specific compartments within the cell. It is therefore possible that the intracellular localization of E6 might change, either as a consequence of differentiation or as a result of exogenous stimuli. Indeed, the PDZ-binding specificity of high-risk E6 has also been shown to be regulated by a cellular pathway: the E6/hDlg interaction is inhibited by PKA phosphorylation of the conserved threonine residue within the PDZ binding domain on E6, and this was also shown to inhibit E6-mediated degradation of hDlg. Indeed, high levels of hDlg could be restored in HPV-18 positive cervical cancer cells by induction of PKA activity (Kühne *et al.*, 2000). Interestingly, PKA regulation of PDZ domain binding was also reported to control the interaction between the K^+ channel Kir2.3 and the PDZ protein PSD-95 (Cohen *et al.*, 1996), and this would suggest that the high-risk E6 proteins might have acquired the PDZ binding domain, with its PKA-associated regulation, from the cellular genome. Its strict conservation among all high-risk E6 proteins is intriguing, and implies a requirement for the virus to finely balance its effects upon hDlg and other PDZ domain-containing proteins. Recent studies have also shown that E6 is phosphorylated by PKN (Gao *et al.*, 2000) and, although the site of phosphorylation has not been mapped, it will nonetheless be extremely interesting to evaluate the effects of this phosphorylation upon E6's other activities.

E6 as a therapeutic target

Since E6 is invariably expressed in cervical lesions and would appear to be responsible for their malignant progression, this protein represents an attractive candidate for developing therapeutic strategies against cervical cancer. Many approaches have been tested to block the expression of E6 in HPV-positive cervical cancer cells, e.g. by selectively inhibiting viral transcription (Goodwin and DiMaio, 2000), or by using antisense constructs (Hamada et al., 1996; von Knebel Doeberitz et al., 1992) or ribozymes (Alvarez-Salas et al., 1998) directed against the polycistronic E6/E7 mRNA. All these approaches cause growth suppression and concomitant reduction of tumorigenicity in vivo, implying the feasibility of reactivating functional tumour suppressor pathways in HPV-positive cells. It can be reasoned that blocking the activity of E6, while retaining that of E7 as a proapoptotic stimulus, might provide a higher therapeutic potential. Since this would be difficult to obtain by targeting the viral mRNAs, since the E6 and E7 genes are expressed together as polycistronic transcripts, this could be done by using peptide aptamers to block the E6 protein in vivo. Indeed, administration of such peptides was found to induce apoptosis of HPV-positive cancer cells (Butz et al., 2000). Thus blocking the E6-mediated degradation of p53 is a major therapeutic goal, since there is strong evidence that the p53-responsive pathways are fully functional in cervical tumour cell lines (Butz et al., 1995, 1999), and reactivating p53 would then bring about growth arrest and/or apoptosis of the HPV transformed cells. However this is unlikely to be universally applicable, since inhibition of E6 induced degradation does not always lead to increased p53 levels. In several cervical cancer cell lines p53 can be stabilized only after additional genotoxic insult,

indicating a lack of intrinsic activation of p53 despite the presence of the viral oncogenes (Mantovani and Banks, 1999). A note of caution should also be added, since targeting the p53/E6AP/E6 complex could interfere with normal E6AP functions, and mutations of E6AP have been implicated in a serious developmental disorder, the Angelman Syndrome (Kishino et al., 1997; Matsuura et al., 1997).

The interactions of E6 with PDZ-proteins are also excellent therapeutic targets, because of their correlation with the malignant progression of HPV-associated disease. Moreover, the binding motif on E6 is small and exposed, and the structures of a number of PDZ domains have been solved (Doyle et al., 1996; Tochio et al., 2000). In addition, the interaction between E6 and its PDZ domain-containing substrates is highly specific, with E6 binding only single PDZ domains on both hDlg and MAGI-1. Therefore it might be feasible to design chemotherapeutic agents, capable of specifically inhibiting the interaction between E6 and this class of targets.

Concluding remarks

During HPV infection E6 plays multiple roles, interfering with several cellular pathways in order to create a favourable environment for viral replication, and neutralizing the cellular surveillance controls that are turned on as the infected cell is unnaturally forced to restart DNA replication. Thus, high-risk E6 blocks apoptosis by targeting p53, Bak and Myc proteins for degradation, however this leaves the cell unprotected from the detrimental effects of DNA mutations. This is confirmed by biochemical and epidemiological studies reporting a synergy between HPV oncogenes and chemical carcinogens in the development of malignancy
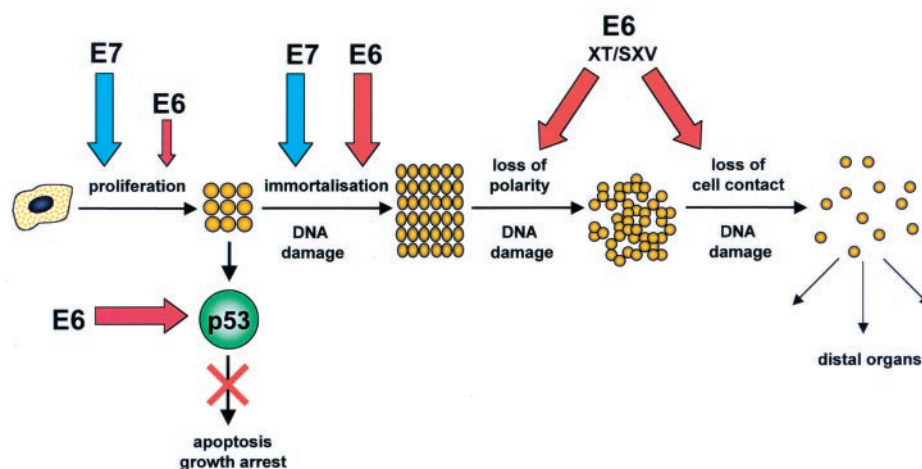

Figure 3 Contribution of HPV E6 to different stages of tumour progression. The role of E6 in tumour promotion is weak compared with that of E7 which actively stimulates cell proliferation. In contrast E6 promotes malignant progression: degradation of p53 overcomes growth arrest and/or apoptosis allowing accumulation of DNA damage, and induction of telomerase contributes towards immortalization. Finally, targeting PDZ-containing proteins through the C-terminal XT/SXV sequence of E6 causes loss of cell polarity and contact, strongly contributing to the malignant phenotype

(Song *et al.*, 2000; Daling *et al.*, 1992). Moreover, E6 inhibits terminal differentiation and senescence, thereby stimulating cell immortalization. Finally, by targeting a class of PDZ domain containing proteins, E6 can also affect cell contact and polarity and, when the pathways regulating these interactions are impaired, either due to mutation or exogenous stimuli, this final loss of control in the immortalized cell can finally lead to an invasive and metastatic phenotype (see Figure 3).

It should be clear, however, that the above picture is far from the 'physiological' outcome of an HPV infection, representing instead the most unfortunate result of a progressive and multifactorial process that only occurs on some occasions. Nonetheless, cervical cancer is an extremely serious disease, representing the third major cause of cancer-related death in women worldwide (Parkin *et al.*, 1999), and the study of the E6 cellular targets has important implications for the development of effective therapeutic strategies.

References

Albrechtsen N, Dornreiter I, Grosse F, Kim E, Wiesmuller and Deppert W. (1999). *Oncogene, 18,* 7706–7717.

Alvarez-Salas L, Cullinan A, Siwkowski A, Hampel A and DiPaolo J. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 1189–1194.

Androphy EJ, Hubbert NL, Schiller JT and Lowy DR. (1987). *EMBO J.,* **6,** 992.

Arbeit J, Münger K, Howley P and Hanahan D. (1993). *Am. J. Pathol.,* **142,** 1187–1197.

Arbeit J, Münger K, Howley P and Hanahan D. (1994). *J. Virol.,* **68,** 4358–4368.

Ashcroft M and Vousden K. (1999). *Oncogene, 18,* 7637–7643.

Askew D, Ashmun R, Simmons B and Cleveland J. (1991). *Oncogene, 6,* 1915–1922.

Baldwin A. (1996). *Annu. Rev. Immunol.,* **14,** 649–683.

Band V, DeCaprio J, Delmolino L, Kulesa V and Sager R. (1991). *J. Virol.,* **65,** 6671–6676.

Band V, Dalal S, Delmolino L and Androphy E. (1993). *EMBO J.,* **12,** 1847–1852.

Banks L, Spence P, Androphy E, Hubbert N, Matlashewski G, Murray A and Crawford L. (1987). *J. Gen. Virol.,* **68,** 1351–1359.

Bannister A and Kouzarides T. (1995). *EMBO J.,* **14,** 4758–4762.

Barbosa M, Lowy D and Schiller J. (1989). *J. Virol.,* **63,** 1404–1407.

Becamel C, Figge A, Poliak S, Dumuis A, Peles E, Bockaert J, Lubbert H and Ullmer C. (2001). *J. Biol. Chem.,* **276,** 12974–12982.

Beer-Romero P, Glass S and Rolfe M. (1997). *Oncogene, 14,* 595–602.

Benton C and Arends MJ. (1996). *Papillomavirus Reviews: Current Research on Papillomaviruses.* C. Lacey (ed). Leeds Medical Information, pp. 271–279.

Bilder D and Perrimon N. (2000). *Nature,* **403,** 676–680.

Bilder D, Li M and Perrimon N. (2000). *Science,* **289,** 113–116.

Boyd J, Malstrom S, Subramaniam T, Venkatesh L, Schaeper U, Elangovan B, D'Sa-Eipper C and Chinnadurai G. (1994). *Cell,* **79,** 341–351.

Burnett A, Barnes W, Johnson J, Grendys E, Willett G, Barter J and Doniger J. (1992). *Gynecol. Oncol.,* **47,** 343–347.

Butz K, Denk C, Ullmann A, Scheffner M and Hoppe-Seyler F. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 6693–6697.

Butz K, Shahabedin L, Geisen C, Spitkovsky D, Ullmann A and Hoppe-Seyler F. (1995). *Oncogene, 10,* 927–936.

Butz K, Whitaker N, Denk C, Ullmann A, Geisen C and Hoppe-Seyler F. (1999). *Oncogene, 18,* 2381–2386.

Caruana G and Bernstein A. (2001). *Mol. Cell Biol.,* **21,** 1475–1483.

Chen J, Hong Y, Rustamzadeh E, Baleja J and Androphy E. (1998). *J. Biol. Chem.,* **73,** 13537–13544.

Chen J, Reid C, Band V and Androphy E. (1995). *Science,* **269,** 529–531.

Cohen N, Brenman J, Snyder S and Bredt D. (1996). *Neuron,* **17,** 759–767.

Comerford SA, Maika SD, Laimins LA, Messing A, Elsasser HP and Hammer RE. (1995). *Oncogene, 10,* 587–597.

Cole ST and Danos O. (1987). *J. Mol. Biol.,* **193,** 599–608.

Cooper K, Herrington CS, Evans MF, Gatter KC and McGee JO. (1993). *J. Pathol.,* **171,** 27–34.

Crook T, Tidy J and Vousden K. (1991). *Cell,* **67,** 547–556.

Daling J, Sherman K, Hislop T, Maden G, Mandelson M, Beckmann A and Weiss N. (1992). *Am. J. Epidemiol.,* **135,** 180–189.

Degenhardt Y and Silverstein S. (2001). *J. Virol.,* **75,** 151–160.

Desaintes C, Hallez P, Van Alphen P and Burney A. (1992). *J. Virol.,* **66,** 325–333.

DiPaolo J, Woodworth C, Popescu MC, Notario V and Doniger J. (1989). *Oncogene, 4,* 395–399.

Dobrosotskaya I and James G. (2000). *Biochem. Biophys. Res. Commun.,* **270,** 903–909.

Dobrosotskaya I, Guy R and James G. (1997). *J. Biol. Chem.,* **272,** 31589–31597.

Donehower L, Harvey M, Slagle B, McArthur M, Montgomery C, Butel J and Bradley A. (1992). *Nature,* **356,** 215–221.

Doorbar J, Foo C, Coleman N, Medcalf L, Hartley O, Prospero T, Napthine S, Sterling J, Winter G and Griffin H. (1997). *Virology,* **238,** 40–52.

Doyle D, Lee A, Lewis J, Kim E, Sheng M and MacKinnon R. (1996). *Cell,* **85,** 1067–1076.

Dürst M, Gallahan D, Jay G and Rhim J. (1989). *Virology,* **173,** 767–771.

Elbel M, Carl S, Spaderna S and Iftner T. (1997). *Virology,* **239,** 132–149.

El-Deiry W, Tokino T, Velculescu V, Levy D, Parsons R, Trent J, Lin D, Mercer W, Kinzler K and Vogelstein B. (1993). *Cell,* **75,** 817–825.

Elmore LW, Hancock AR, Chang SF, Wang XW, Chang S, Callahan CP, Geller DA, Will H and Harris CC. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 14707–14712.

Elston R, Napthine S and Doorbar J. (1998). *J. Gen. Virol.,* **79,** 371 – 374.

Etscheid BG, Foster SA and Galloway DA. (1994). *Virology,* **205,** 583 – 585.

Farrow S, White J, Martinou I, Raven T, Pun K, Grinham C, Martinou J and Brown C. (1995). *Nature,* **374,** 731 – 733.

Fortunato EA and Spector DH. (1998). *J. Virol.,* **72,** 2033 – 2039.

Foster S, Demers W, Etscheid B and Galloway D. (1994). *J. Virol.,* **68,** 5698 – 5705.

Freedman D and Levine AJ. (1998). *Mol. Cell Biol.,* **18,** 7288 – 7293.

Gao Q, Kumar A, Srinivasan S, Singh L, Mukai H, Ono Y, Wazer D and Band V. (2000). *J. Biol. Chem.,* **275,** 14824 – 14830.

Gao Q, Singh L, Kumar A, Srinivasan S, Wazer D and Band V. (2001). *J. Virol.,* **75,** 4459 – 4466.

Gao Q, Srinivasan S, Boyer S, Wazer D and Band V. (1999). *Mol. Cell Biol.,* **19,** 733 – 744.

Gardiol D, Kühne C, Glausinger B, Lee S, Javier R and Banks L. (1999). *Oncogene,* **18,** 5487 – 5496.

Gewin L and Galloway DA. (2001). *J. Virol.,* **75,** 7198 – 7201.

Glaunsinger B, Lee S, Thomas M, Banks L and Javier R. (2000). *Oncogene,* **19,** 5270 – 5280.

Goodman R and Smolik S. (2000). *Genes Dev,* **14,** 1553 – 1577.

Goodwin E and DiMaio D. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 12513 – 12518.

Griep AE, Herber R, Jeon S, Lohse JK, Dubielzig RR and Lambert PF. (1993). *J. Virol.,* **67,** 1373 – 1384.

Gross-Mesilaty S, Reinstein E, Bercovich B, Tobias K, Schwartz A, Kahana C and Ciechanover A. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 8058 – 8063.

Hamada K, Sakaue M, Alemany R, Zhang W, Horio Y, Roth J and Mitchell M. (1996). *Gynecol. Oncol.,* **63,** 219 – 227.

Harper J, Adami G, Wei N, Keyomarsi K and Elledge S. (1993). *Cell,* **75,** 805 – 816.

Hawley-Nelson P, Vousden K, Hubbert N, Lowy D and Schiller J. (1989). *EMBO J.,* **8,** 3905 – 3910.

Hengstermann A, Linares L, Ciechanover A, Whitaker N and Scheffner M. (2001). *Proc. Natl. Acad. Sci. USA,* **98,** 1218 – 1223.

Herber R, Liem H, Pitot H and Lambert P. (1996). *J. Virol.,* **70,** 1873 – 1881.

Honda R, Tanaka H and Yasuda H. (1997). *FEBS Lett.,* **420,** 25 – 27.

Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, Johnson R and Karin M. (1999). *Science,* **284,** 316 – 320.

Huibregtse J, Scheffner M and Howley P. (1991). *EMBO J.,* **10,** 4129 – 4135.

Huibregtse J, Scheffner M and Howley P. (1993a). *Mol. Cell. Biol.,* **13,** 775 – 784.

Huibregtse J, Scheffner M and Howley P. (1993b). *Mol. Cell. Biol.,* **13,** 4918 – 4927.

Hurlin P, Kaur P, Smith P, Perez-Reyes N, Blanton R and McDougall J. (1991). *Proc. Natl. Acad. Sci. USA,* **88,** 570 – 574.

Ide N, Hata Y, Nishioka H, Hirao K, Yao I, Deguchi M, Mizoguchi A, Ishimori H, Tokino T, Nakamura Y and Takai Y. (1999). *Oncogene,* **18,** 7810 – 7815.

Isaacs JS, Barrett JC and Weissman BE. (1999). *Mol. Carcinog.,* **24,** 70 – 77.

Ishidate T, Matsumine A, Toyoshima K and Akiyama T. (2000). *Oncogene,* **19,** 365 – 372.

Jackson S and Storey A. (2000). *Oncogene,* **19,** 592 – 598.

Jackson S, Harwood C, Thomas M, Banks L and Storey A. (2000). *Genes Dev.,* **14,** 3065 – 3073.

Kanda T, Watanabe S, Zanma S, Sato H, Furuno A and Yoshiike K. (1991). *Virology,* **185,** 536 – 543.

Kao W, Beaudenon S, Talis A, Huibregtse J and Howley P. (2000). *J. Virol.,* **74,** 6408 – 6417.

Kemp C, Donehower L, Bradley A and Balmain A. (1993). *Cell,* **74,** 813 – 822.

Kessis T, Slebos R, Nelson W, Kastan M, Plunkett B, Han S, Lorincz A, Hedrick L and Cho K. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 3988 – 3992.

Kim E, Niethammer M, Rothschild A, Nung Yan Y and Sheng M. (1995). *Nature,* **378,** 85 – 88.

Kim S. (1997). *Curr. Opin. Cell. Biol.,* **9,** 853 – 859.

Kinzler K and Vogelstein B. (1996). *Cell,* **87,** 159 – 170.

Kishino T, Lalande M and Wagstaff J. (1997). *Nat. Genet.,* **15,** 70 – 73.

Kiyono T, Foster S, Koop J, McDougall J, Galloway D and Klingelhutz A. (1998). *Nature,* **396,** 84 – 88.

Kiyono T, Hiraiwa A, Fujita M, Hayashi Y, Akiyama T and Ishibashi M. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 11612 – 11616.

Klingelhutz A, Foster S and McDougall J. (1996). *Nature,* **380,** 79 – 82.

König C, Roth J and Dobbelstein M. (1999). *J. Virol.,* **73,** 2253 – 2262.

Krajewski S, Krajewska M and Reed J. (1996). *Cancer Res.,* **56,** 2849 – 2855.

Kühne C and Banks L. (1998). *J. Biol. Chem.,* **273,** 34302 – 34309.

Kühne C, Gardiol D, Guarnaccia C, Amenitsch H and Banks L. (2000). *Oncogene,* **19,** 5884 – 5891.

Kukimoto I, Aihara S, Yoshiike K and Kanda T. (1998). *Biochem. Biophys. Res. Commun.,* **249,** 258 – 262.

Kurman R, Schiffman M, Lancaster W, Reid R, Jenson A, Temple G and Lorincz A. (1988). *Am. J. Obstet. Gynecol.,* **159,** 293 – 296.

Lechner M and Laimins L. (1994). *J. Virol.,* **68,** 4262 – 4273.

Lechner M, Mack D, Finicle A, Crook T, Vousden K and Laimins L. (1992). *EMBO J.,* **11,** 3045 – 3052.

Lee D, Lee B, Kim J, Kim W and Choe J. (2000a). *J. Biol. Chem.,* **275,** 7045 – 7051.

Lee S, Glausinger B, Mantovani F, Banks L and Javier R. (2000b). *J. Virol.,* **74,** 9680 – 9693.

Lee S, Weiss R and Javier R. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 6670 – 6675.

Lepik D, Ilves I, Kristjuhan A, Maimets T and Ustav M. (1998). *J. Virol.,* **72,** 6822 – 6831.

Li X and Coffino P. (1996). *J. Virol.,* **70,** 4509 – 4516.

Lie AK, Skarsvag S, Skomedal H, Haugen OA and Holm R. (1999). *Int. J. Gynecol. Pathol.,* **18,** 5 – 11.

Lin J, Chen J, Elenbaas B and Levine AJ. (1994). *Genes Dev.,* **8,** 1235 – 1246.

Liu Y, Chen J, Gao Q, Dalal S, Hong Y, Mansur C, Band V and Androphy E. (1999). *J. Virol.,* **73,** 7297 – 7307.

Liu Z, Ghai J, Ostrow RS, McGlennen RC and Faras AJ. (1994). *Virology,* **201,** 388 – 396.

Lowe S, Jacks T, Houseman D and Ruley E. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 2026 – 2030.

Lue R, Marfatia S, Branton D and Chishti A. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 9818 – 9822.

Lyons R and Moses H. (1990). *Eur. J. Biochem.,* **187,** 467 – 473.

Mancini A, Koch A, Stefan M, Niemann H and Tamura T. (2000). *FEBS Lett.,* **482,** 54 – 58.

Mantovani F and Banks L. (1999). *Oncogene,* **18,** 3309 – 3315.

Mantovani F Massimi P and Banks L. (2001). *J. Cell Sci.* (in press).

Marcello A, Massimi P, Banks L and Giacca M. (2000). *J. Virol.*, **74,** 9090 – 9098.

Marfatia S, Morais Cabral J, Lin L, Hough C, Bryant P, Stolz L and Chishti A. (1996). *J. Cell. Biol.*, **135,** 753 – 766.

Marte B and Downward J. (1997). *Trends Biochem. Sci.*, **22,** 355 – 358.

Massimi P, Pim D, Bertoli C, Bouvard V and Banks L. (1999). *Oncogene*, **18,** 7748 – 7754.

Matlashewski G, Banks L, Pim D and Crawford L. (1986). *Eur. J. Biochem.*, **154,** 665 – 672.

Matsumine A, Ogai A, Senda T, Okumura N, Satoh K, Baeg G, Kawahara T, Tobayashi S, Okada M, Toyoshima K and Akyama T. (1996). *Science*, **272,** 1020 – 1023.

Matsuura T, Sutcliffe J, Fang P, Galjaard R, Jiang Y, Benton C, Rommens J and Beaudet A. (1997). *Nat. Genet.*, **15,** 74 – 77.

Menon AG, Rutter JL, von Sattel JP, Synder H, Murdoch C, Blumenfeld A, Martuza RL, von Deimling A, Gusella JF and Houseal TW. (1997). *Oncogene*, **14,** 611 – 616.

Münger K, Phelps WC, Bubb V, Howley PM and Schlegel R. (1989). *J. Virol.*, **63,** 4417 – 4421.

Nakagawa S and Huibregtse J. (2000). *Mol. Cell Biol.*, **20,** 8244 – 8253.

Nakagawa S, Watanabe S, Yoshikawa H, Taketani Y, Yoshiike K and Kanda T. (1995). *Virology*, **212,** 535 – 542.

Nees M, Geoghean JM, Hyman T, Frank S, Miller L and Woodworth CD. (2001). *J. Virol.*, **75,** 4283 – 4296.

Pan H and Griep A. (1994). *Genes Dev.*, **8,** 1285 – 1299.

Pan H and Griep A. (1995). *Genes Dev.*, **9,** 2157 – 2169.

Parkin D, Pisani P and Ferlay J. (1999). *Int. J. Cancer*, **80,** 827 – 841.

Patel D, Huang S, Baglia L and McCance. (1999). *EMBO J.*, **18,** 5061 – 5072.

Pecoraro G, Morgan D and Defendi V. (1989). *Proc. Natl. Acad. Sci. USA*, **86,** 563 – 567.

Peng Y, Breiding D, Sverdrup F, Richard J and Androphy E. (2000). *J. Virol.*, **74,** 5872 – 5879.

Pietenpol J, Holt J, Stein R and Moses R. (1990a). *Proc. Natl. Acad. Sci. USA*, **87,** 3758 – 3762.

Pietenpol J, Stein R, Moran E, Yaciuk P, Schlegel R, Lyons R, Pittelkow M, Münger K, Howley P and Moses H. (1990b). *Cell*, **61,** 777 – 785.

Pim D, Storey A, Thomas M, Massimi P and Banks L. (1994). *Oncogene*, **9,** 1869 – 1876.

Pim D, Massimi P and Banks L. (1997). *Oncogene*, **15,** 257 – 264.

Pim D, Thomas M, Javier R, Gardiol D and Banks L. (2000). *Oncogene*, **19,** 719 – 725.

Polakis P. (1999). *Curr. Opin. Genet. Dev.*, **9,** 15 – 21.

Polyak K, Xia Y, Zweler J, Kinzler K and Vogelstein B. (1997). *Nature*, **389,** 300 – 305.

Querido E, Morisson M, Chu-Pham-Dang H, Thirlwell S, Boivin D and Branton P. (2001). *J. Virol.*, **75,** 699 – 709.

Reuver SM and Garner CC. (1998). *J. Cell. Sci.*, **111,** 1071 – 1080.

Reznikoff C, Belair C, Savelieva E, Zhai Y, Pfeifer K, Yeager T, Thompson K, DeVries S, Bindley C and Newton M. (1994). *Genes Dev.*, **8,** 2227 – 2240.

Ronco L, Karpova A, Vidal M and Howley P. (1998). *Genes Dev.*, **12,** 2061 – 2072.

Ruppert J and Stillmann B. (1993). *Mol. Cell Biol.*, **13,** 3811 – 3820.

Sastry S and Burridge K. (2000). *Exp. Cell. Res.*, **261,** 25 – 36.

Sastry S and Horwitz A. (1996). *Dev. Biol.*, **180,** 455 – 467.

Scheffner M, Werness B, Huibregtse J, Levine A and Howley P. (1990). *Cell*, **63,** 1129 – 1136.

Schneider-Gädicke A, Kaul S, Schwarz E, Gausepohl H, Frank R and Bastert G. (1988). *Cancer Res.*, **48,** 2969 – 2974.

Schwarz E, Freese U, Gissmann L, Mayer W, Roggenbuck B, Stremlau A and zur Hausen H. (1985). *Nature*, **314,**

Schwarz S, Rosa J and Scheffner M. (1998). *J. Biol. Chem.*, **273,** 12148 – 12154.

Sedman SA, Barbosa MS, Vass WC, Hubbert NL, Haas JA, Lowy DR and Schiller JT. (1991). *J. Virol.*, **65,** 4860 – 4866.

Sherman L and Schlegel R. (1996). *J. Virol.*, **70,** 3269 – 3279.

Sherman L, Jackman A, Itzhaki H, Stoppler M, Koval D and Schlegel R. (1997). *Virology*, **237,** 296 – 306.

Snijders P, van Duin M, Walboomers J, Steenbergen R, Risse E, Helmerhorst T, Verheijen R and Meijer C. (1998). *Cancer Res.*, **58,** 3812 – 3818.

Somasundaram K and El-Deiry WS. (1997). *Oncogene*, **14,** 1047 – 1057.

Song S, Pitot H and Lambert P. (1999). *J. Virol.*, **73,** 5887 – 5893.

Song S, Liem A, Miller J and Lambert P. (2000). *Virology*, **267,** 141 – 150.

Songyang Z, Fanning A, Fu C, Xu J, Marfatia S, Chishti A, Crompton A, Chan A, Anderson J and Cantley L. (1997). *Science*, **275,** 73 – 77.

Steegenga W, Riteco N, Jochemsen AG, Fallaux F and Bos J. (1998). *Oncogene*, **16,** 349 – 357.

Steller M, Zou Z, Schiller J and Baserga R. (1996). *Cancer Res.*, **56,** 5087 – 5091.

Storey A and Banks L. (1993). *Oncogene*, **8,** 919 – 924.

Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Leigh I, Matlashewski G and Banks L. (1998). *Nature*, **393,** 229 – 234.

Suzuki T, Ohsugi Y, Uchida-Toita M, Akiyama T and Yoshida M. (1999). *Oncogene*, **18,** 5967 – 5972.

Talis A, Huibregtse J and Howley P. (1998). *J. Biol. Chem.*, **273,** 6439 – 6445.

Thanos D and Maniatis T. (1995). *Cell*, **83,** 1091 – 1100.

Thomas M and Banks L. (1998). *Oncogene*, **17,** 2943 – 2954.

Thomas M and Banks L. (1999). *J. Gen. Virol.*, **80,** 1513 – 1517.

Thomas M, Massimi P, Jenkins J and Banks L. (1995). *Oncogene*, **10,** 261 – 268.

Thomas M, Glaunsinger B, Pim D, Javier R and Banks L. (2001). *Oncogene*, **20,** 5431 – 5439.

Tochio H, Huang F, Li M, Bredt D and Zhang M. (2000). *J. Mol. Biol.*, **295,** 225 – 237.

Tong X and Howley P. (1997). *Proc. Natl. Acad. Sci. USA*, **94,** 4412 – 4417.

Turner CE. (2000). *J. Cell. Sci.*, **113,** 4139 – 4140.

Ullmer C, Schmuck K, Figge A and Lubbert H. (1998). *FEBS Lett*, **424,** 63 – 68.

von Knebel Doeberitz M, Rittmuller C, zur Hausen H and Dürst M. (1992). *Int. J. Cancer*, **51,** 831 – 834.

Veldman T, Horikawa I, Barret J and Schlegel R. (2001). *J. Virol.*, **75,** 4467 – 4472.

Wathelet M, Lin C, Parekh B, Ronco L, Howley P and Maniatis T. (1998). *Mol. Cell*, **1,** 507 – 518.

Wazer DL, Liu XL, Chu Q, Gao Q and Band V. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 3687 – 3691.

Weaver B, Prasanna Kumar K and Reich N. (1998). *Mol. Cell Biol.*, **18,** 1359 – 1386.

Weis K, Griffiths G and Lamond A. (1994). *J. Biol. Chem.*, **269,** 19142 – 19150.

White A, Livanos E and Tlsty T. (1994). *Genes Dev.,* **8,** 666–677.

Wilcock D and Lane DP. (1991). *Nature,* **349,** 429–431.

Woods D, Hough C, Peel D, Callaini G and Bryant PJ. (1996). *J. Cell Biol.,* **134,** 1469–1482.

Woodworth C, Doniger J and DiPaolo J. (1989). *J. Virol.,* **63,** 159–164.

Wu X and Levine A. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 3602–3606.

Wu X, Hepner K, Castelino-Prabhu S, Do D, Kaye M, Yuan X, Wood J, Ross C, Sawyers C and Whang Y. (2000a). *Proc. Natl. Acad. Sci. USA,* **97,** 4233–4238.

Wu Y, Dowbenko D, Spencer S, Laura R, Lee J, Gu Q and Lasky L. (2000b). *J. Biol. Chem.,* **275,** 21477–21485.

Zhang J, Rose B, Thompson C, Jarrett C, Russell P, Houghton R and Cossart Y. (1995). *Gynecol. Oncol.,* **57,** 170–177.

Zimmermann H, Degenkolbe R, Bernard H and O'Connor M. (1999). *J. Virol.,* **73,** 6209–6219.

zur Hausen H and Schneider A. (1987). *The Papillomaviruses.* Salzman NP, Howley PM (eds). Plenum Publishing Corp.: New York, pp. 245–263.